EXHIBIT E

Human Estrogen Receptor Ligand Activity Inversion Mutants: Receptors That Interpret Antiestrogens as Estrogens and Estrogens as Antiestrogens and Discriminate among Different Antiestrogens

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The estrogen receptor (ER) is a transcription factor whose activity is normally activated by the hormone estradiol and inhibited by antiestrogen. It has been found that certain mutational changes in the activation function-2 region in the hormone-binding domain of the human ER result in ligand activity inversion mutants, *i.e.* receptors that are now activated by antiestrogen and inhibited by estrogen. The ER point mutant L540Q is activated by several antiestrogens (the more pure antiestrogens ICI 164,384 and RU 54,876 or the partial antiestrogen *trans*-hydroxytamoxifen) but not by estradiol. The presence of the F domain and an intact activation function-1 in the A/B domain are required for this activity, as is the DNA-binding ability of the receptor. This inverted ligand activity is observed with several estrogen-responsive promoters, both simple and complex; however, the activating ability of antiestrogens is observed only in some cells, highlighting the important role of cell-specific factors in ligand interpretation. The introduction of two additional amino acid changes close to 540 results in receptors that are still not activated by estradiol but are now able to distinguish between partial antiestrogens (which remain agonistic) and pure antiestrogens (which show a greatly reduced stimulatory activity). These ligand activity inversion mutants remain stable in cells in the presence of the antiestrogen ICI 164,384, as does a related ER mutant receptor that shows the normal, wild type ER ligand activity profile in which ICI 164,384 is transcriptionally inactive. Thus, the presence of adequate levels of mutant ER may be necessary but not sufficient for ICI 164,384 to elicit transcriptional activity. These findings highlight the means by which the carboxyl-terminal region in domain E functions to interpret the activity of a ligand, and they demonstrate that rather minimal changes in the ER can result in receptors with inverted response to antiestrogen and estrogen. Such point mutations, if present in estrogen target cells, would result in antiestrogens being seen as growth stimulators, rather than suppressors, with potentially detrimental consequences in terms of breast cancer treatment with antiestrogens. (Molecular Endocrinology 10: 230–242, 1996)

INTRODUCTION

The estrogen receptor (ER), a member of a large nuclear hormone receptor superfamily, binds steroidal or nonsteroidal ligands and functions as a hormone-activated transcription regulator. The ER, like other members of this receptor superfamily, has two domains that have been highly conserved during evolution, domains involved in DNA binding and hormone binding. Deletion and mutational analysis has enabled the mapping of four important functions of the receptor, namely, ligand binding, dimerization, DNA binding, and transcription activation. The amino-terminal A/B region of the receptor exhibits a hormone-independent transactivation function; the central region (domain C) is principally involved in receptor DNA interaction; and the carboxyl-terminal domains (E/F) are structurally and functionally complex and contain hormone/antihormone binding, dimerization, and

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hormone-dependent transactivation functions. (For reviews, see Refs. 1–4). Upon binding estrogen, the receptor binds to estrogen response element (ERE) DNA, often located in the 5'-flanking region of estrogen-responsive genes. The estrogen-occupied receptor is then thought to interact with transcription factors and other components of the transcription complex to modulate gene expression (5).

The actions of estrogens are antagonized by antiestrogens, which bind to the ER in a manner competitive with estrogen, but fail to effectively activate gene transcription. Antiestrogens vary in their biological actions. Certain ones such as tamoxifen (used widely in the treatment of hormone-dependent human breast cancer and uterine cancer) act as partial agonists/antagonists, with the degree of agonist or antagonist activity dependent upon the cell type and promoter context (6–8). Other antiestrogens, such as ICI 164,384, ICI 182,780, and RU 54,876 appear to be more pure/complete antagonists (9–11).

Several groups have reported that ER-antiestrogen complexes differ from ER-estrogen complexes in receptor conformation, DNA binding, and recruitment of transcription factors necessary to effectively activate gene transcription (12–19). These data suggest that the hormone and antihormone complexes display different conformations, which are dependent on the nature of the ligand. Presumably, the transcription apparatus reads an antiestrogen-receptor complex differently from an estrogen-receptor complex, a process that may involve the interaction of factors exclusive for one complex or the other. Nevertheless, the precise molecular mechanism of action of antiestrogen *vs.* estrogen remains unclear.

In our studies examining structure-activity relationships in the ER that involve identifying residues in the hormone-binding domain (HBD) important for ligand binding and transactivation functions of the receptor, we have been particularly interested in understanding the mechanisms by which the ER discriminates between estrogen and antiestrogen ligands. Site-directed mutagenesis of selected residues in the ER and region-specific chemical mutagenesis of the ER HBD enabled us to identify a region close to the C terminus of domain E, near C530, that appears to be important in hormone-dependent transcription activation and the discrimination between estrogens and antiestrogens (3, 20–22). Studies of Pakdel and Katzenellenbogen (21) and Danielian *et al.* (23) have shown that altering selected amino acids near C530 changed the binding affinity for estrogens but not for antiestrogens. In addition, we have reported on several ER mutants with alterations in the carboxyl-terminal portion of the HBD that were transcriptionally inactive with E_2, yet bound hormone and also functioned as potent dominant negative ERs, efficiently suppressing the activity of wild type ER occupied by E_2 (24, 25).

In studies reported here, we show that one of these dominant negative ERs, which is transcriptionally inactive in response to E_2, can be activated by antiestrogen in some cell contexts. Surprisingly, antiestrogen-stimulated transcriptional activity of this L540Q receptor is suppressed by estrogen, indicating that this mutant shows switched or inverted ligand activity. We present data on the roles of activation functions-1 and -2 (AF-1 and AF-2, respectively) and the role of domain F of the ER in this antiestrogen stimulation and also provide data showing that further changes of amino acids near leucine 540, in the AF-2 region, enable the receptor to discriminate between the pure and partial agonist/antagonist categories of antiestrogen. Such mutations, if naturally occurring in breast tumors, could explain generalized antiestrogen resistance as well as tamoxifen resistance, yet sensitivity to more pure antiestrogens such as ICI 164,384.

RESULTS

The ER Mutant L540Q Shows an Inverted Response to Ligands and Is Transcriptionally Activated by Antiestrogens but not Estradiol

We have shown previously that the ER point mutant L540Q is transcriptionally inactive in response to estrogen and that it further acts as a potent dominant negative ER, inhibiting the activity of the estradiol-occupied wild type ER when both proteins are expressed in the same cells (24, 25). Shown in Fig. 1A is the fact that, as reported previously, the L540Q ER is incapable of being activated by E_2, although it binds E_2 with wild type affinity (24). Interestingly, we observe that although this mutant is not transcriptionally active with E_2, it can be stimulated by either the partial antiestrogen *trans*-hydroxytamoxifen (TOT) or the pure antiestrogen ICI 164,384 (Fig. 1, B and C).

In these studies (Fig. 1), an ER-negative human breast cancer cell line, MDA-MB-231, was transfected with an estrogen-responsive reporter construct, $(ERE)_2$-pS2-CAT, along with an expression vector for wild type (wt) ER or the L540Q mutant ER. Cells were then monitored for chloramphenicol acetyltransferase (CAT) activity after treatment with varying concentrations of E_2, TOT, or ICI 164,384. Shown in panel A is the fact that the wt ER exhibits strong (200-fold) transcriptional activation by E_2. TOT evokes a weaker, dose-dependent stimulation of the wt ER that reaches a level approximately 20% that of E_2 (panel B); and the pure antiestrogen ICI 164,384 shows no stimulation of wt ER at any concentration tested (10^{-10} M to 10^{-7} M; panel C).

By contrast, the L540Q mutant ER exhibits an unusual phenotype—the ability to respond to both antiestrogens but not to E_2. While E_2 was not able to stimulate transcriptional activation of L540Q ER, TOT showed stimulation of the L540Q receptor similar to that observed with wt ER; and ICI 164,384, while not able to activate wt ER, stimulated transcriptional activity of L540Q from the reporter plasmid $(ERE)_2$-pS2-CAT to levels 22% of that achieved with the wt ER in

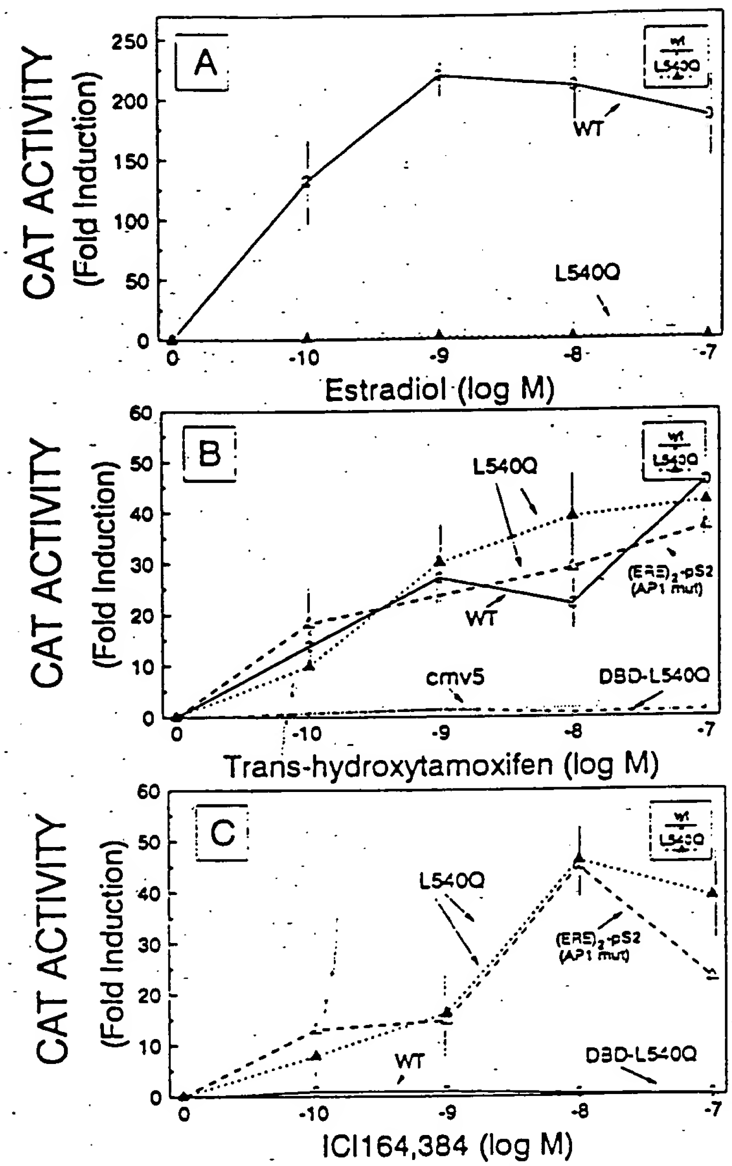


Fig. 1. L540Q ER Responds to Both Partial and Pure Antiestrogens but Not to E_2

The response of wt ER and L540Q ER to E_2, TOT, and ICI 164,384 was determined in 231 cells. Cells were transfected with the $(ERE)_2$-pS2-CAT reporter plasmid, either with wt or L540Q ER expression vector and a β-galactosidase internal reporter to correct for transfection efficiency. They were then treated for 24 h with varying concentrations of E_2 (panel A), TOT or (panel B), or ICI 164,384 (panel C). Cell extracts were prepared and analyzed for β-galactosidase and CAT activity as described in *Materials and Methods*. In certain cases (panels B and C) cells were transfected with the $(ERE)_2$-pS2-CAT reporter plasmid containing a mutated AP-1 site [$(ERE)_2$-pS2(AP-1 mut)-CAT], or with the empty expression vector missing the ER cDNA (cmv5; panel B), or with an expression vector for the L540Q ER lacking the ability to bind EREs (DBD-L540Q). Transcriptional activation is reported as fold stimulation over the basal level of CAT activity in cells transfected with the reporter plasmid only, which is set at 1. Values are the means and range from two separate experiments. If error bars are not shown, they were smaller than the symbols.

response to E_2. We observed a similar stimulation of transcriptional activity of L540Q in the presence of another pure antiestrogen, ICI 182,780 (data not presented). The response of L540Q to the antiestrogens TOT and ICI 164,384 occurred in a dose-dependent manner, with half-maximal stimulation occurring for TOT and ICI 164,384 at 0.5 nM and 1.9 nM, respectively, reflecting the different relative binding affinities of these ligands for the ER (26, 27).

Since putative binding sites for AP-1 have been identified within the backbone of small pUC plasmids, and it has been shown that TOT can have regulatory effects on transcription through AP-1 sites in some cells (28), we repeated the experiments with the reporter plasmid $(ERE)_2$-pS2-CAT in which we mutated the AP-1 site, denoted $(ERE)_2$-pS2(AP1 mut)-CAT. As shown in panels B and C, the response to both TOT and ICI 164,384 was maintained, indicating that the

AP-1 site is not responsible for the transcriptional effect.

We also tested the ability of a further modified form of the L540Q receptor to stimulate transcriptional activity. Mutant L540Q lacking the ability to bind estrogen response element (ERE) DNA, denoted DBD-L540Q, was generated and found not to respond to any concentration of TOT or ICI 164,384 (Fig. 1, B and C). Likewise, cells transfected only with the empty expression vector (pCMV5, lacking the L540Q ER cDNA) showed no transcriptional activity (Fig. 1B). These findings indicate that transcriptional activation of L540Q by the antiestrogens TOT and ICI 164,384 is most likely mediated via receptor binding to ERE DNA.

E_2 Is Able to Suppress the Transcriptional Activity of the L540Q ER Elicited by Antiestrogens

Since we have shown that the mutant L540Q is able to bind E_2 with wild type affinity without being stimulated by it (20), we examined whether the transcriptional activation of L540Q by ICI 164,384 and TOT could be inhibited by E_2. As shown in Fig. 2, E_2 showed a dose-dependent repression of TOT-mediated (panel A) and ICI 164,384-mediated transactivation by L540Q (panel B); 50% repression was achieved at about 10^{-8} M E_2 (~10-fold less E_2 than TOT or ICI 164,384). Thus the L540Q mutant shows a reversed or inverted ligand transcriptional response to antiestrogens *vs.* estrogen when compared with the wt ER.

Assessment of Promoter Specificity in the Inverted Ligand Activity Response of the L540Q ER

Since it is well known that the ER shows cell and promoter specificity in the activation of gene transcription (*e.g.* Refs. 6–8), we examined the transcriptional activation of other gene constructs, namely $(ERE)_2$-TATA-CAT, containing a simple promoter, and $(ERE)_2$-$PR_{proximal}$-CAT, containing the complex progesterone receptor gene proximal promoter. As was observed in

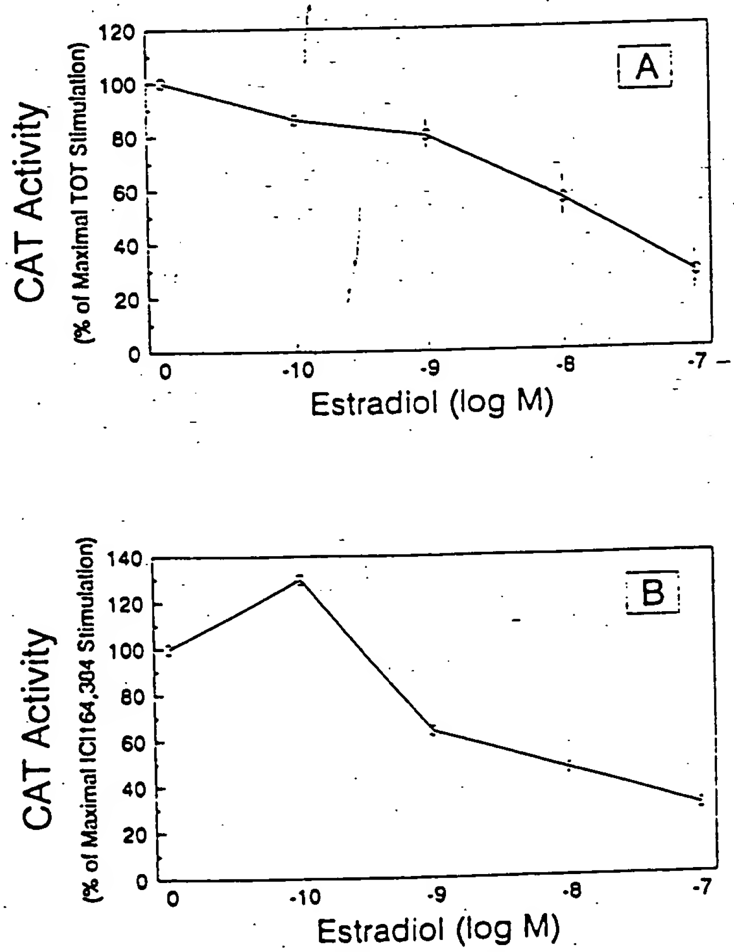


Fig. 2. Repression of Antiestrogen-Stimulated Transcriptional Activity of L540Q ER by E_2

231 Cells were transfected with the $(ERE)_2$-pS2-CAT reporter plasmid, the L540Q ER expression vector, and a β-galactosidase internal reporter to correct for transfection efficiency. They were then treated for 24 h with (A) 10^{-7} M TOT or (B) 10^{-7} M ICI 164,384 and varying concentrations of E_2. Cell extracts were prepared and analyzed for β-galactosidase and CAT activity as described in *Materials and Methods*. Values are the means and range from two separate experiments.

Fig. 1 for the estrogen-responsive pS2 promoter-containing reporter gene construct, wt ER showed stimulation in response to E_2 but not in response to ICI 164,384 (Fig. 3, A and B). The L540Q ER failed to show transcriptional activity in response to E_2, but ICI 164,384 evoked transcriptional activity with both the TATA and the progesterone receptor gene promoters to levels that were approximately 20–30% that observed for E_2 stimulation of wt ER (Fig. 3, A and B). Therefore, the ability of ICI 164,384 but not E_2 to stimulate L540Q activity was also evident with these different promoter constructs.

Relative Roles of AF-1, AF-2, and the F Domain in the Transcriptional Response of the L540Q Receptor to Antiestrogens

The partial agonistic activity of tamoxifen is believed to be correlated with the activity of AF-1, located in the A/B domain of the ER (6). We have previously shown

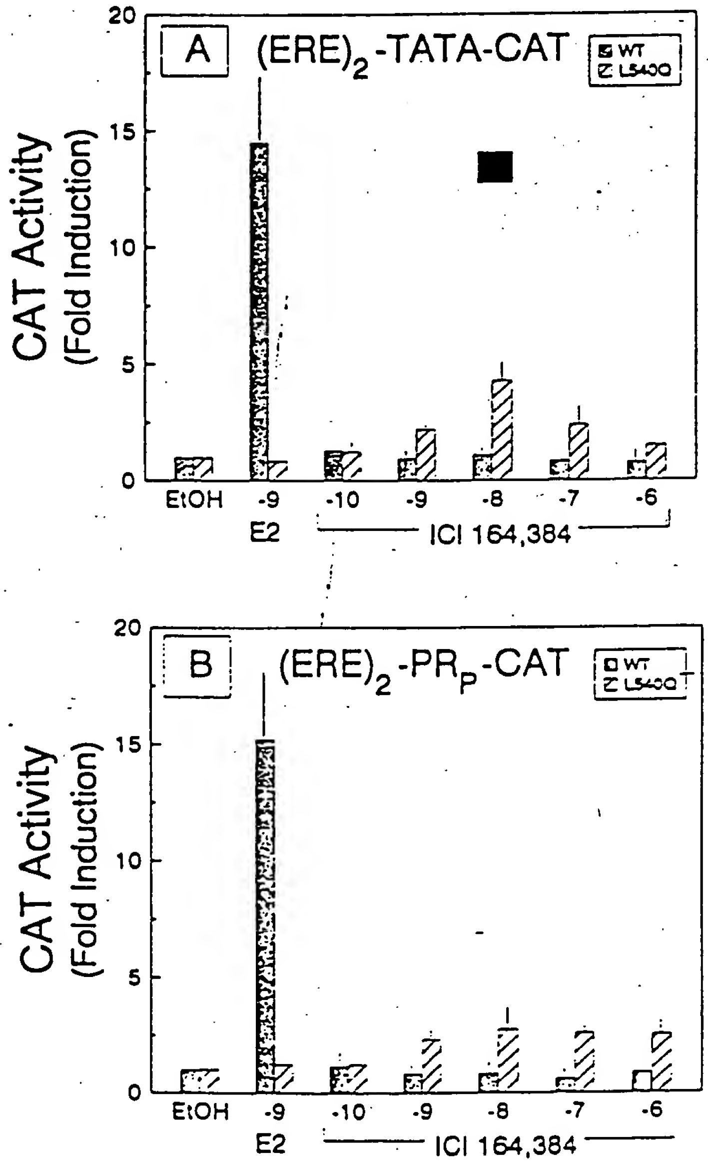


Fig. 3. Examination of Promoter Specificity in the Magnitude of Antiestrogen Stimulation of the L540Q ER

The response of wt ER and L540Q ER to E_2 and ICI 164,384 was determined in 231 cells transfected with (A) $(ERE)_2$-TATA-CAT or (B) $(ERE)_2$-$PR_{proximal}$-CAT reporter plasmids, either with wt ER or L540Q ER expression vector, and a β-galactosidase internal reporter to correct for transfection efficiency. They were then treated for 24 h with 10^{-9} M E_2 or the indicated concentrations of ICI 164,384. Cell extracts were prepared and analyzed for β-galactosidase and CAT activity as described in *Materials and Methods*. Transcriptional activation is reported as fold stimulation over the basal level of CAT activity in cells transfected with the reporter plasmid only, which is set at 1. Basal CAT activity was the same for the L540Q and wt ER. Values are the means and range from two separate experiments.

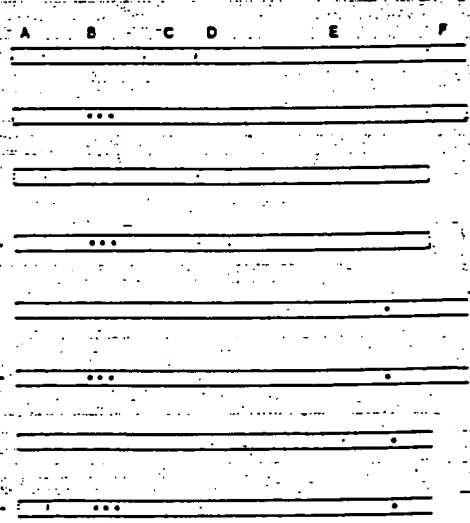
the F domain to have an important modulatory role in mediating response to antiestrogens (7), perhaps by enhancing AF-1 function and/or the interactions of AF-1 with AF-2. We therefore examined the relative roles of AF-1, AF-2, and the F-domain in antiestrogen/estrogen-stimulated activity of the L540Q and wt ER by introducing mutations that disrupt either AF-1 or AF-2 function, or by deleting the C-terminal F domain.

The response of the wild type receptor to E_2, TOT, or ICI 164,384 is shown in Fig. 4 (line 1). Impairment of AF-1 function by introduction of three point mutations that change serines 104, 106, and 118, sites of ER phosphorylation (29), to alanine, had little effect on E_2-mediated transcriptional activity but nearly fully eliminated the stimulation of wt ER by TOT, consistent with the currently held belief that antiestrogens exert their agonistic activity largely through AF-1 (line 2). As we reported recently for wt ER (7), removal of the F domain also eliminated the agonistic activity of TOT but had no effect on E_2-mediated transcriptional activity of the wt ER (line 3). Combination of the triple serine mutation and F domain deletion from the wt ER resulted in a receptor showing no response to TOT, as expected, and only minimally reduced in its response to E_2 (line 4).

As shown in Fig. 4, line 5, the L540Q receptor showed substantial response to TOT and ICI 164,384 but no response to E_2. Introduction of the triple serine-to-alanine mutations in domain B markedly reduced the response to TOT and ICI 164,384 (line 6), implying that antiestrogen stimulation of the L540Q receptor requires an intact AF-1 function. Remarkably, deletion of the F domain in the L540Q protein (line 7) resulted in a dramatic activation of the receptor by E_2 to levels about 70% that of the wild type response to E_2, while resulting in a nearly complete loss of response to the antiestrogens TOT and ICI 164,384. The combination of the triple mutations in domain B with F domain deletion in the L540Q receptor resulted in a phenotype as expected, namely good stimulation by E_2 and no stimulation by TOT or ICI 164,384. Thus, the stimulation of the L540Q receptor by antiestrogens requires AF-1 function and the presence of the F domain. In addition, the nonresponsiveness of the L540Q mutant to E_2 is strongly dependent on the presence of the F domain as deletion of this domain restores E_2 responsiveness of the L540Q mutant.

Amino Acid Changes in the AF-2 Region of the L540Q Receptor Generate Ligand Activity Inversion Mutants Which Distinguish among Different Antiestrogens

To further examine the role of AF-2 in the activity of the L540Q receptor, we introduced mutations of two additional amino acid residues (E542A and D545A) in this region. The residues L540, E542, and D545



	Fold Induction		
	E2 (10^{-9}M)	TOT (10^{-7}M)	ICI (10^{-7}M)
(1) wild type	167 ± 24	22.9 ± 4.7	0.6 ± 0.1
(2) S104A, S106A, S118A	150 ± 26	2.7 ± 0.7	0.8 ± 0.3
(3) ΔF	161 ± 30	1.2 ± 0.5	1.0 ± 0.3
(4) S104A, S106A, S118A-ΔF	113 ± 19	1.4 ± 0.2	0.6 ± 0.2
(5) L540Q	2.2 ± 0.5	39.5 ± 9.9	36.2 ± 6.3
(6) S104A, S106A, S118A-L540Q	2.9 ± 1.0	8.5 ± 0.4	8.0 ± 0.3
(7) L540Q-ΔF	109 ± 21	9.1 ± 3.4	0.6 ± 0.2
(8) S104A, S106A, S118A-L540Q-ΔF	99.3 ± 13	2.2 ± 0.6	0.7 ± 0.3

Fig. 4. Point Mutations in the A/B Domain and Deletion of the F Domain Decrease the Agonistic Activity of Both Partial and Pure Antiestrogens with the L540Q ER

The response of wt and mutant ERs to E_2, TOT, and ICI 164,384 was determined in 231 cells. Cells were transfected with the $(ERE)_2$-pS2-CAT reporter plasmid, either with wt ER or mutant ER expression vector, and a β-galactosidase internal reporter to correct for transfection efficiency. They were then treated for 24 h with E_2 (10^{-9} M), TOT (10^{-7} M), or ICI 164,384 (10^{-7} M). Cell extracts were prepared and analyzed for β-galactosidase and CAT activity as described in *Materials and Methods*. Transcriptional activation is reported as fold stimulation over the basal level of CAT activity in cells transfected with the reporter plasmid only, which is set at 1. Values are the mean ± SE from at least three separate experiments.

are fully conserved across all ER species from human to *Xenopus* and trout (30–32) and are considered to be important in AF-2 function (8, 23). The AF-2 region has been mapped to encompass amino acids 534–548 of the human ER, and mutations in this region have been shown to affect receptor transcriptional activity but not hormone binding (20, 32). The triple mutant (L540Q, E542A, D545A) and double mutant (E542A, D545A), like the L540Q ER (20), bound E_2 with wild type affinity [dissociation constant (K_d) 0.2–0.5 nM], as reported for analogous mutants of the mouse ER (32). However, only the double mutant showed a transcriptional response to E_2. As seen in Fig. 5, the E542A, D545A receptor showed a profile of ligand response very similar to that of wt ER in that transcriptional activity was stimulated by E_2 and TOT (E_2 more effectively than TOT) but not by the two more pure antiestrogens ICI 164,384 and RU 54,876. The L540Q receptor showed good response to all the antiestrogens (TOT, ICI 164,384, and RU 54,876). However, in the L540Q, E542A, D545A triple mutant receptor, response to TOT was preserved (and could be suppressed by E_2 as shown for the L540Q ER in Fig. 2) while the response to ICI 164,384 was completely lost and the response to RU 54,876 was considerably reduced (Fig. 5).

Cell Dependence in the Agonistic Activity of Antiestrogen-L540Q Receptor Complexes

The ability of the L540Q receptor, when occupied by antiestrogen, to evoke transcriptional activity was only observed in some cells. As shown in Table 1, and in *Results* above, the L540Q-antiestrogen complex showed transcriptional activity in 231 breast cancer cells with the several ERE-containing promoter reporter constructs tested (pS2, $PR_{proximal}$, and TATA), while no response was evoked by E_2. In another breast cell line, MCF-10A cells, antiestrogen, but not estrogen, also stimulated the $(ERE)_2$-pS2-CAT promoter reporter construct. However, this gene construct, as well as several others tested, were not activated by either E_2 or antiestrogen in Chinese hamster ovary (CHO) cells or 3T3 mouse fibroblast cells (Table 1). These observations, indicating that the ability of the antiestrogen-L540Q receptor complex to function as a transcriptional activator is markedly influenced by cell context, is not unexpected, since it has become increasingly clear that ER stimulation of transcription by hormones depends not only on the nature of the ligand and on the receptor (whether variant or wild type), but also shows marked cell and promoter specificity.

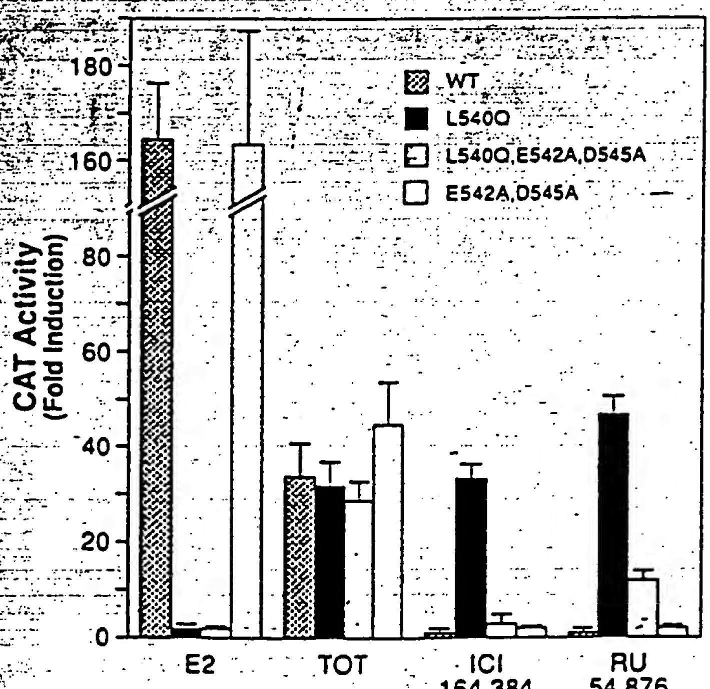


Fig. 5. Point Mutants in the AF-2 Region with Differential Response to Partial and Pure Antiestrogens

The response of the point mutant L540Q, the triple amino acid mutant L540Q, E542A, D545A, and the double amino acid mutant E542A, D545A to E_2, TOT, ICI 164,384, and RU54,876 was determined in 231 cells. Cells were transfected with the $(ERE)_2$-pS2-CAT reporter plasmid, either with L540Q ER or L540Q, E542A, D545A ER expression vector, and a β-galactosidase internal reporter to correct for transfection efficiency. They were then treated for 24 h with E_2 (10^{-9} M), TOT (10^{-7} M), ICI 164,384 (10^{-7} M), or RU 54,876 (10^{-7} M). Cell extracts were prepared and analyzed for β-galactosidase and CAT activity as described in *Materials and Methods*. Values are the means and range from two separate experiments.

Table 1. Transcriptional Activity of the ER Mutant L540Q with E_2 or Antiestrogen (ICI 164,384 or TOT) in Different Cells and Promoter Contexts

Cell	Promoter[a]	Response to E_2	Response to ICI 164,384 or TOT
MDA-MB-231	pS2	[illegible]	[illegible]
	$PR_{proximal}$	[illegible]	[illegible]
	TATA	[illegible]	[illegible]
MCF-10A	pS2	[illegible]	[illegible]
CHO	pS2	[illegible]	[illegible]
	TATA[b]	[illegible]	[illegible]
	Vitellogenin[b]	[illegible]	[illegible]
3T3	pS2	[illegible]	[illegible]
	$PR_{proximal}$[b]	[illegible]	[illegible]

−, No transcriptional response. +, transcriptional stimulation; positive responses with ICI 164,384 or TOT were approximately 20% relative to E_2 stimulation of wt ER, which is set at 100%.

[a] Promoter-reporter gene constructs contain two consensus EREs in the $(ERE)_2$-pS2-CAT, $(ERE)_2$-$PR_{proximal}$-CAT and $(ERE)_2$-TATA-CAT constructs and one consensus ERE in the ERE-vitellogenin-CAT construct.

[b] Ref. 52.

The Ligand Activity Inversion Mutant Proteins Remain Stable in the Presence of ICI 164,384, as Does a Related Mutant Receptor, but ICI 164,384 Only Activates Transcription of Some of These Mutants and in Only Some Cells

Since it had not been anticipated that the L540Q receptor would show activation by antiestrogens, we examined the levels of the L540Q protein in cells treated with various concentrations of ICI 164,384, TOT, or E_2, using Western blot analysis. A slight increase in the levels of L540Q mutant ER was observed in response to ICI 164,384 (at 10^{-7} M or 5×10^{-7} M) compared with the levels of L540Q in 231 cells treated with control vehicle only (Fig. 6). In these experiments, cells containing wt ER and treated with 1×10^{-7} M ICI 164,384 exhibited the expected decrease in ER protein level. Therefore, the L540Q protein appeared to be resistant to the usual ER destabilizing effects of ICI 164,384. The increase in L540Q levels in response to ICI 164,384 was also observed in CHO cells (Fig. 6) and is, therefore, a generalized phenomenon for this particular mutant protein. However, the transcriptional response to ICI 164,384 observed with L540Q in 231 cells was not obtained in CHO cells (Table 1). Thus, the agonist activity of ICI 164,384 that is mediated by L540Q ER in some cells appears to require cell-specific factors and is not simply a consequence of the stability of this mutant ER in the presence of ICI 164,384.

Analysis of ER levels by immunoblot (Fig. 6) also indicated that the proteins L540Q, E542A, D545A and E542A, D545A were affected similarly as was the L540Q protein in the presence of ICI 164,384—no decrease in the levels of the three mutant receptor proteins was seen in response to ICI 164,384. However, the transcriptional response of these three receptors to ICI 164,384 was very different (Fig. 5), consistent with a model in which the presence of adequate levels of ER is necessary but not sufficient for ICI 164,384 to be able to evoke transcriptional activity. Western blot analysis also showed that the effects of E_2 and TOT on levels of the wt and mutant ERs were very similar, with E_2 decreasing ER levels and TOT slightly increasing ER levels (data not shown). Therefore, although the mutant proteins remain stable in the presence of ICI 164,384 while the wild type protein is degraded more rapidly in the presence of this compound (Fig. 6, and Refs. 13 and 33), the mutant receptors show marked differences in their transcriptional response and interpretation of antiestrogen ligands.

DISCUSSION

Ligand Interpretation by the ER: Influence of Ligand Type, Receptor Domains, and Cell Context

Our findings identify an unusual ER phenotype that exhibits a response to ligands that is the inverse of that seen with wt ER. The L540Q receptor interprets antiestrogens as estrogens, and E_2, being able to bind to this mutant ER but unable to activate transcription (20, 24), can suppress the stimulation by antiestrogen. Our studies also reveal that additional perturbations of nearby amino acids in the AF-2 region of the ER result in receptors that are able to discriminate among different antiestrogens. The triple point mutant receptor L540Q, E542A, D545A is not activated by the pure antiestrogen ICI 164,384 and is only weakly stimulated by the antiestrogen RU 54,876, but it still retains its agonistic, stimulatory response to TOT. This L540Q, E542A, D545A triple mutant receptor is, to the best of our knowledge, the first ligand discrimination point mutant of the ER capable of discriminating among different antiestrogens. Thus, the interpretation of a compound as being an agonist or antagonist of a biological response is strongly determined by the nature of the receptor, namely, whether it is variant or wild type.

This laboratory has had a longstanding interest in the identification and analysis of ligand discrimination mutants of the ER. Previous studies by us (3, 20, 21) using site-directed and random chemical mutagenesis, with screening in yeast and mammalian cell systems, identified several critical regions in the HBD in which changes resulted in receptors altered in their estrogen/antiestrogen binding and transcriptional activation, documenting that the ER itself clearly can discriminate between these two categories of ligands. However, our previously identified ligand discrimina-

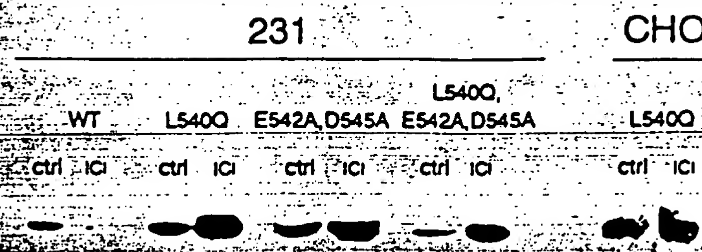


Fig. 6. Immunoblot Determination of wt and Mutant ER Expression Levels in 231 Cells and in CHO Cells

Cells were transfected with wt or mutant ER expression plasmids as described in *Materials and Methods* and were treated with 0.1% ethanol control vehicle (ctrl) or 10^{-7} M ICI 164,384 for 24 h. Cell extracts containing 100 μg protein were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with ER-specific monoclonal antibody, H222, a second bridging antibody, and [^{125}I]protein A.

tion mutants showed altered binding and response to estrogens and antiestrogens, but they did not discriminate among different antiestrogens. In addition, they did not show inverted ligand activity; although all antiestrogens became more effective antagonists, they were still seen as antagonist ligands. Interestingly, the ER mutants investigated in this report are able to discriminate among different antiestrogens, suggesting that this region, known to encompass at least a critical portion of the AF-2 region of the ER, is extremely important in ligand interpretation and receptor-ligand transcriptional activity. Studies by McDonnell and co-workers (8, 34) using different *in vitro* models have also emphasized the distinct biologies of different antiestrogens.

The agonistic response of L540Q ER to TOT and ICI 164,384 was eliminated either by compromising AF-1 activity or deleting the F domain (Fig. 4), providing evidence for the roles of both of these regions in the transcriptional response of the ligand-receptor complexes. Likewise, the loss of agonistic activity of TOT and ICI 164,384 in the L540Q receptor missing the F domain, and the loss of TOT, but not E_2, stimulatory activity in the wt ER with its F domain deleted, indicates that the response to the antiestrogens depends on an intact F region.

Remarkably, L540Q response to E_2 could be restored by removal of the F domain. This highlights that the F domain has the potential to play an important role in maintaining or regulating the conformation of the AF-2 region, which includes amino acid 540, in a way that affects ligand response. The complete lack of responsiveness of the L540Q receptor to E_2 is apparently enforced by domain F, as its deletion restores transcriptional responsiveness. Indeed, there is already considerable evidence that this region is important in interactions with ER-associated proteins (35–38) that may function as transcriptional coactivators.

Using several different promoters, we have observed inverted or reversed ligand discrimination by the L540Q and L540Q, E524A, D545A mutant ERs. Although ICI 164,384 and TOT evoked substantial agonistic activity on the three different promoters examined, the activity was maximally only about 25–30% of that evoked by E_2 with the wt ER. The fact that the magnitude of transcriptional activation was never as great as that achieved with the wt ER in the presence of E_2 implies that the conformation of the antiestrogen-L540Q receptor is not perfect for full transcriptional activity. These findings contrast with those of Mahfoudi *et al.* (39), who reported stimulation by antiestrogen of a mutant mouse ER roughly similar to that achieved with E_2 stimulation of wild type receptor; this observation may reflect cell and/or promoter differences or the fact that in their HeLa cell and reporter gene system, wild type receptor evoked only a 3- to 4-fold increase in CAT activity in the presence of E_2, compared with our approximately 200-fold, making their system possibly less sensitive and quantitative.

Our observations highlight that ERs containing mutations in the AF-2 region, namely mutation of the highly conserved leucine at amino acid 540 or mutation of amino acids 542 and 545, show protein stabilization such that they are not turned over rapidly in the presence of ICI 164,384. Related leucine mutations in the mouse ER have also been reported to remain stable in the presence of ICI 164,384 (39). However, we show that the transcriptional activation of the L540Q receptor by ICI 164,384 appears not to be due solely to the stabilization of the receptor but appears to require cell-specific factors in addition. This is indicated by our experiments conducted in CHO cells, wherein no activation of L540Q in the presence of ICI 164,384 was obtained (Table 1) despite the stabilization of the L540Q receptor and its failure to be turned over rapidly by ICI 164,384 (Fig. 6). Also, the fact that the E542A, D545A double mutant remains stable in the presence of ICI 164,384, but is not transcriptionally activated by this ligand, indicates that the presence of the mutant ER protein may be necessary but is clearly not sufficient to elicit ICI 164,384 agonism.

The C-terminal region of ER, which we have shown to be important in cell-specific ligand interpretation, is a region that is highly conserved across the nuclear receptor superfamily (32). Therefore, it is of note that alterations in this region in the glucocorticoid receptor and progesterone receptor have been found to affect discrimination between hormone and antihormone li-

gands (40, 41) and to result in human progesterone receptors that are activated by the antiprogestin RU 486 but not by progestin (41).

Tamoxifen Stimulation/Tamoxifen Resistance in Breast Cancer and the Possible Involvement of ER Ligand Activity Inversion Mutants

Our studies show that it is the receptor that interprets a ligand as an agonist or antagonist, but that this is also critically dependent on the particular cell context. We have previously reported on mutants near C530 altered in their binding affinity for estrogen and/or antiestrogen. These mutants showed altered transcriptional response to these two categories of ligands as a consequence of differences in their binding of these ligands (21). The mutants shown here, in a slightly more C-terminal portion of domain E, show wild type binding affinity for both estrogens and antiestrogens and bind to ERE DNA (Ref. 24 and data not presented). The ligand interpretation, therefore, appears to be a consequence of the changes in the AF-2 region most likely related to interactions with transcription factors and ER-associated proteins that function to modulate receptor activity (35–38). Our findings highlight that a change of a single amino acid (L540Q) in this AF-2 region is able to alter receptor conformation so that the receptor interprets antiestrogens as agonists and estrogen as an antagonist.

It is an intriguing possibility that the reversed pharmacological phenotype seen in the L540Q receptor may explain some of the tamoxifen resistance, or even tamoxifen stimulation, of breast tumors that are observed under long-term tamoxifen exposure in experimental tumors and possibly in humans as well. These types of mutations might explain the benefit sometimes observed upon tamoxifen withdrawal in some women who have been taking tamoxifen (42, 43). Also, receptors like the triple mutant L540Q, E542A, D545A could explain the phenotype of tamoxifen resistance yet sensitivity to suppression by the more pure antiestrogen ICI 164,384 (44). Certainly, however, future studies will be needed to determine whether such mutations do occur naturally in some breast cancers.

The studies reported here highlight that the interpretation of a hormone receptor complex depends critically on the nature of the ligand and the receptor. While the ER itself can discriminate between estrogen and antiestrogen and can distinguish among different antiestrogens, the consequence of this ligand discrimination depends on the cell context, so that the L540Q receptor exposed to antiestrogen was transcriptionally productive only in some, but not all, cells. This suggests a critical role for cell-specific factors and other ER-associated proteins in biological response to the ligand-ER complex.

MATERIALS AND METHODS

Chemicals and Materials

Cell culture media were purchased from GIBCO (Grand Island, NY). Calf serum was from Hyclone Laboratories (Logan, UT) and FCS from Sigma Chemical Co. (St. Louis, MO). Radioinert 17β-estradiol was obtained from Sigma Chemical Co. [2,4,5,6-^{3}H]estradiol (90 Ci/mmol) and [dichloroacetyl-1,2-^{14}C] chloramphenicol (50–60 Ci/mmol) were from DuPont, NEN Research Products (Wilmington, DE). The antiestrogens ICI 164,384 and TOT were provided by Alan Wakeling and Zeneca Pharmaceuticals (Macclesfield, England). The antiestrogen RU 54,876 was kindly provided by Francois Nique and Roussel UCLAF.

Plasmid Construction

All cloning was done using standard techniques. When necessary to make termini compatible, 3'- and 5'-overhangs generated by restriction digestion were blunted with T4 DNA polymerase and the Klenow fragment of *Escherichia coli* DNA polymerase, respectively. The generation of point mutants and the deletion of DNA fragments were confirmed by dideoxy chain termination DNA sequencing. Other manipulations were confirmed by restriction digest analyses.

ER Mutants

The L540Q mutant was generated by random chemical mutagenesis as described previously (20). The ΔF mutant hER was generated by site-directed mutagenesis as described previously (45). The phosphorylation mutant, S104A/S106A/S118A, was also generated by site-directed mutagenesis as described previously (29). Both wt and mutant ERs were subcloned into the eukaryotic expression vector pCMV5 as described previously (20).

S104A/S106A/S118A-ΔF was constructed by digesting the mutant ER, S104/S106A/S118A, with *Hind*III/*Not*I to generate two inserts, 260 bp and 822 bp, which were then ligated into the *Hind*III-digested ΔF mutant ER. S104A/S106A/S118A-L540Q was constructed by ligating the two inserts from the *Hind*III/*Not*I digest of S104/S106A/S118A into the *Hind*III-digested L540Q. L540Q-ΔF was constructed by *Xba*I/*Bsp*MI digest of L540Q, followed by religation with *Bsp*MI-partially digested ΔF ER. S104A/S106A/S118A-L540Q-ΔF was then generated by replacing the *Hind*III/*Not*I fragments of L540Q-ΔF mutant ER with the *Hind*III/*Not*I fragments of S104/S106A/S118A. The DNA binding deficient L540Q mutant, DBD-L540Q, was constructed by replacing the *Eag*I/*Eag*I fragment of L540Q with the *Eag*I/*Eag*I fragment of pCMV-hEG82 that contains three point mutations in domain C that render the ER unable to bind to ERE DNA (46).

The mutants L540Q, E542A, D545A and E542A, D545A were made by site-directed mutagenesis (47), by first inserting the 1.8-kb *Bam*HI ER-containing fragment from pCMV-L540Q or pCMV5-ER into the *Bam*HI site of pBluescript II SK$^-$, creating L540Q-BSII-SK$^-$ (pKE101) and ER-BSII-SK$^-$ (pKE109). The E542A and D545A mutations were then engineered into L540Q or wt ER by oligo-directed mutagenesis (47), using the oligonucleotides 5'-CCTGCAGCTCGCGATGCTAGCAGCCCACCGCCTAC-3' and 5'-GTAGGCGGTGCGCAGCTAGCATCGCGAGCAGCAGG-3'. The 1.8-kb ER-containing fragments were then cloned back into pCMV5, generating pCMV5-L540Q, E542A, D545A, and pCMV5-E542A, D545A.

Reporter Gene Constructs

The estrogen responsive plasmid, $(ERE)_2$-pS2-CAT, was constructed as described previously (48). To generate $(ERE)_2$-

pS2(AP1 mut)-CAT, we first mutated the AP1 site (located about 300 bp from the stop codon of the gene for CAT) in pTZ-tk-CAT (49). pTZ-tk(AP1 mut)-CAT was made by site-directed mutagenesis using the mutagenic oligonucleotide 5'-TTACTAAACACAGCAGTACTCAAAAAACTTAGCAA-3' annealed to single-stranded DNA generated from pTZ-tk-CAT using the f1 origin of replication that it contains. $(ERE)_2$-pS2(AP1 mut)-CAT was then generated by replacing the NaeI/*Nco*I fragment of pTZ-tk(AP1 mut)-CAT with the NaeI/*Nco*I fragment of $(ERE)_2$-pS2-CAT.

The plasmid $(ERE)_2$-$PR_{proximal}$-CAT containing the distal promoter of the rat progesterone reporter gene was prepared as described (49). The reporter plasmid $(ERE)_2$-TATA-CAT (50) was kindly provided by David Shapiro of the University of Illinois (Urbana, IL). The plasmid pCMVβ (Clonetech, Palo Alto, CA) which contains the β-galactosidase gene was used as an internal control for transfection efficiency in all experiments. The plasmid pTZ19, used as a carrier DNA, was provided by Byron Kemper of the University of Illinois.

Cell Culture and Transfections

MDA-MB-231 human breast cancer cells were maintained in Leibovitz's L15 medium with 10 mM HEPES, 5% calf serum, 100 U penicillin/ml, 100 μg streptomycin/ml, 25 μg gentamycin/ml, 6 ng bovine insulin/ml, 3.75 ng hydrocortisone/ml, and 16 μg glutathione/ml. Cells were then grown in MEM plus phenol red supplemented with 5% charcoal dextran-treated calf serum (CDCS) for 2 days. These cells were seeded for transfection at 3×10^6 cells per 100-mm dish in Improved MEM (IMEM) minus phenol red containing 5% CDCS and were given fresh medium about 30 h after plating. All media included penicillin (100 U/ml) and streptomycin (100 μg/ml).

MCF-10A human breast epithelial cells were obtained from Dr. Robert J. Pauley (Michigan Cancer Foundation, Detroit, MI). MCF-10A cells were maintained in DMEM/Nutrient mixture F-12 Ham's (DME/F12) with 15 mM HEPES without phenol red, supplemented with 1.344 g/liter sodium bicarbonate, 73 mg/liter L-glutamine, 55 mg/liter sodium pyruvate, 5% horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5 μg/ml fungizone, 10 μg/ml bovine insulin, 0.5 μg/ml hydrocortisone, 20 μg/ml epidermal growth factor, 0.1 μg/ml cholera toxin. Cells were then grown in DME/F12 supplemented with 5% CDCS for 5 days. MCF-10A cells were seeded for transfection at 3×10^6 cells per 100-mm dish in IMEM minus phenol red containing 5% CDCS and were given fresh medium about 30 h after plating. All media included penicillin (100 U/ml) and streptomycin (100 μg/ml).

231 and MCF-10A cells were transfected by the $CaPO_4$ coprecipitation method 16 h later with 2 μg $(ERE)_2$-pS2-CAT, 10 μg $(ERE)_2$-$PR_{proximal}$-CAT reporter plasmid, or 10 μg $(ERE)_2$-TATA-CAT, 100 μg ER expression vector, 800 μg pCMVβ β-galactosidase internal control plasmid, and pTZ19 carrier DNA. Cells remained in contact with the precipitate for 5 h and were then subjected to a 2.5-min glycerol shock (20% in IMEM minus phenol red plus 5% CDCS). Cells were rinsed with HBSS and given fresh media with or without hormones.

CHO cells were maintained in phenol-red free DME/F12 tissue culture medium supplemented with 10% charcoal dextran-treated FCS (CDFCS); 100 U/ml penicillin (GIBCO), and 100 μg/ml streptomycin (GIBCO). They were plated at 1.8×10^5 cells per 60-mm dish, maintained at 37 C in a humidified 5% CO_2 atmosphere for 48 h, and transfected by calcium phosphate coprecipitation method (51). In transactivation assays, 60-mm plates were treated with 0.4 ml of DNA precipitate containing 1 μg $(ERE)_2$-pS2-CAT or 1.6 μg $(ERE)_2$-TATA-CAT reporter plasmid, 0.2 μg pCH110 β-galactosidase internal control plasmid, 2 μg ER expression vector, and 6.2 μg pTZ carrier DNA. In all cases, cells remained in contact with the precipitate for 12–16 h and were then subjected to a 2-min glycerol shock (20% glycerol in HBSS). Plates were rinsed, given 4 ml fresh media, and treated with hormones. 3T3 mouse fibroblast cells were grown and transfected exactly as described (7).

All cells were harvested 24 h after hormone treatment, and extracts were prepared in 200 μl 250 mM Tris, pH 7.5, using three freeze-thaw cycles. β-Galactosidase activity was measured (51) to normalize for transfection efficiency among plates. CAT assays were performed as previously described (51).

Immunoblots

Whole-cell extracts were prepared from the cells by resuspending the cell pellet from a 100-mm dish of cells in 200 μl 50 mM Tris-HCl (pH 7.5)-1 mM EDTA-1 mM NaCl and then incubating on ice for 20 min and centrifugation at $14,000 \times g$. Extracts were fractionated on polyacrylamide gels under reducing conditions. Proteins were transferred from SDS gels to nitrocellulose and subjected to Western immunoblot analysis with anti-ER monoclonal antibodies as described (20).

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